NOTE TO FILE
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Subject: Male sterile Radicchio rosso (red hearted chicory)

Keywords: Radicchio rosso, *Cichorium intybus*, red hearted chicory, *Bacillus amyloliquefaciens barnase (ribonuclease)* gene, male sterility, *Streptomyces hygroscopicus bar* gene, phosphinothricin acetyltransferase (PAT) protein, phosphinothricin (glufosinate) tolerant, herbicide tolerant, kanamycin resistance (*kan*^{r}) gene, aminoglycoside 3'-phosphotransferase II (APH(3') II).

Background

In a submission dated May 20, 1997, Bejo Zaden provided summary information to support their safety and nutritional assessment of a new hybrid seed production system (SeedLink™) in Radicchio rosso (red hearted chicory). This hybrid seed production system is based on the development by Bejo Zaden of transgenic male sterile Radicchio rosso lines containing transformation events RM3-3, RM3-4 or RM3-6.

Intended Effect and Food Use

According to Bejo Zaden, hybrid Radicchio rosso varieties have several advantages over non-hybrid varieties, including pure seed quality with high germination rate, high productivity and uniformity of the crop. Although Radicchio generally produces seed by cross-pollination, occasionally self-pollination occurs. Traditionally, the breeding of hybrid Radicchio crops has utilized self-incompatibility between the parental lines. However, the alleles regulating this self-incompatibility often appear to be unstable, resulting in some self-pollination (15%-20%) in the parental lines during seed production.

According to Bejo Zaden, their transgenic male sterile Radicchio rosso lines contain the *barnase* gene which encodes the enzyme barnase, a ribonuclease derived from *Bacillus amyloliquefaciens*. The *barnase* gene is selectively expressed in the tapetal cell layer of the anthers during early anther development. The tapetal cell layer plays a vital nutritive role during pollen formation. Expression of the barnase enzyme in the tapetal cell layer disrupts production of RNA, and thus, expression of protein in these cells. This disruption in protein expression, in essence, destroys the tapetal cell layer, rendering the anthers incapable of producing viable pollen grains. This inability to produce viable pollen grains renders the plant male sterile and provides reliable pollination control.

The *barnase* gene is linked to the selectable marker gene coding for phosphinothricin tolerance (*bar*), which encodes the enzyme phosphinothricin acetyltransferase (PAT). PAT was derived from *Streptomyces hygroscopicus* and confers tolerance to the herbicide phosphinothricin (glufosinate ammonium) by specifically acetylating phosphinothricin. Since the *barnase* gene and *bar* gene are physically linked, these genes will segregate as a single locus. Consequently, the male sterile line can be maintained by cross-pollinating with wild-type plants followed by the application of the phosphinothricin herbicide. The design of the insertion confines the use of the herbicide application strictly to breeding and selection purposes. The hybridization technique used for the production of the hybrids is based on the genetic male sterility of one of the parental lines. In the hybrid product the new traits (male sterility and phosphinothricin-tolerance) are expected to be present in 50% of the plants.

Radicchio rosso (red hearted chicory) belongs to the *Cichorium* family which also includes endive, Belgian endive and Pan di Zucchero (green hearted chicory). Industrial chicory, cultivated for its inulin production, does not belong to this group of vegetable chicories. The Radicchio rosso crop is a vegetable crop commercially grown for human consumption usually as a component of fresh mixed salads.

Molecular Alterations and Characterization

Bejo Zaden used the *Agrobacterium tumefaciens*-mediated plant transformation system to transfer new DNA containing three genes and appropriate regulatory elements into the genome of Radicchio rosso cells. The plasmid pGV825 which contains genes for male sterility (*barnase*), kanamycin resistance (*kan*^{r}*; also known as aph(3') II, aphA-2, npt II and neo*^{r}), and phosphinothricin tolerance (*bar*) along with appropriate regulatory elements within the border sequences of the T-DNA (transfer DNA) was used by the firm as an intermediate cloning vector. The firm produced a co-integrative plasmid, pTTM8RE, by introducing the intermediate vector pGV825 carrying the genes of interest into an appropriate *Agrobacterium tumefaciens* host strain containing the acceptor plasmid pGV2260, from which the T-region has been removed. By homologous recombination, the genes of interest from the intermediate vector pGV825 were transferred into the acceptor plasmid pGV2260, producing the co-integrative plasmid, pTTM8RE. With this modified *Agrobacterium tumefaciens* containing the pTTM8RE co-integrative plasmid, Bejo Zaden transformed Radicchio rosso cells.

Bejo Zaden confirmed that the *barnase*, *kan*^{r}, and *bar* genes were integrated stably into the Radicchio rosso genome in lines RM3-3, RM3-4 and RM3-6 by performing Southern blot analyses on three generations of plants. These analyses demonstrated that lines RM3-3 and RM3-4 contained one copy of transferred DNA incorporated into the genome in the same configuration as in the plasmid pTTM8RE. For the line RM3-6, two copies had originally incorporated into the genome. One copy was complete and present in all three generations tested and was incorporated into the genome in the same configuration as in the plasmid pTTM8RE. However, the other copy was rearranged resulting in a non-functional *bar* gene and was lost due to segregation after the first generation.

To determine if the integrated DNA was solely from within the T-DNA borders, the firm performed polymerase chain reaction (PCR) analysis. Three different primers were used simultaneously with genomic DNA from each of the transgenic lines, RM3-3, RM3-4 and RM3-6, for left border analysis. Two primers were homologous with internal sequences of the T-DNA near the left border and one was homologous to plasmid sequences outside of the T-DNA border. For all three transgenic lines, only one PCR product was observed. The size of this PCR product was consistent with the size expected for DNA produced from the two primers homologous to internal sequences of the T-DNA. According to Bejo Zaden, the results indicate that for the three transgenic plant lines, sequences beyond the left border from the plasmid are not present in the plant genomes.

Each gene has an appropriate promoter, a region in the DNA that regulates expression of the gene, so that the gene products will be expressed in the desired tissue of the plant. The *bar* gene is controlled by the promoter PssuAra and expressed as a fusion protein with an N-terminal transit peptide sequence for translocation to the chloroplasts. The *kan*^{r} gene is regulated by the nopaline-synthase-promoter, providing expression of aminoglycoside 3'-phosphotransferase (APH(3') II; also known as neomycin phosphotransferase (NPT II)) throughout the plant. The *barnase* gene is under control of the pTA29 promoter which selectively expresses barnase in the tapetal cell layer of the anthers during early anther development.

Expressed Proteins

Using Northern Blot analyses, Bejo Zaden was able to detect mRNA from *bar* and *kan*^{r} genes in leaf, head and root samples, but was unable to detect mRNA from the *barnase* gene in heterologous vegetative and floral organ systems. Bejo Zaden considered their inability to detect mRNA transcripts from the *barnase* gene consistent with the restrictive promoter used for expression of the *barnase* gene which restricts expression to one region (tapetum cells of the pollen sac) during a short period of development (during anther development). Using enzymatic activity assays of two to four samples, Bejo Zaden determined the protein expression levels of the PAT protein and APH(3') II protein in the heads and leaves of Radicchio. The average expression levels for the PAT protein are 0.04% of the total protein for line RM3-3, 0.06% in line RM3-4 and 0.065% in line RM3-6 for samples from the head and 0.63% for line RM3-3, 0.45% in line RM3-4 and 0.61% in line RM3-6 for samples from the leaves. The average expression levels for the APH(3') II protein are 0.022% of the total protein in line RM3-3, 0.018% in line RM3-4 and 0.021% in line RM3-6 for samples from the head and 0.005% in line RM3-3, 0.005% in line RM3-4, and 0.015% in line RM3-6 for samples from the leaves.

Allergenic and Toxic Potential

No endogenous toxicants of Radicchio were reported by Bejo Zaden. For barnase, APH(3') II and PAT, Bejo Zaden conducted a protein sequence search for homology to other polypeptides in the HIVAA7, PIR42 and Swiss-Prot30 databases. Bejo Zaden reports that no significant homology to known allergens was observed. The PAT protein showed no homology with known toxic peptides. Furthermore, the PAT protein is rapidly degraded in simulated gastric fluid (SGF).

Protein could not be detected upon immediate sampling of the reaction mixture using standard SGF. Bejo Zaden referenced published literature which document the finding that allergenic food proteins are typically resistant to proteolytic degradation. Bejo Zaden referenced a published report entitled "Safety assessment of the bacterium-derived recombinant phosphinothricin acetyltransferase (PAT) protein" by the State Institute for Quality Control of Agricultural Products (RIKILT-DLO) in Wageningen, The Netherlands (Ref. 1). The authors of this report conclude that experimental evidence has demonstrated that the PAT protein does not have characteristics similar to allergenic proteins, such as heat or proteolytic stability, high concentrations in food or homology to known food allergens or toxins.

APH(3') II is regulated as a food additive under 21 CFR 173.150 for use as a processing aid in the development of new varieties of tomato, oilseed rape and cotton. FDA evaluated APH(3') II as a food additive in response to a petition filed by Calgene, Inc. At the time the petition was filed, the use of APH(3') II as a processing aid in the development of new plant varieties was new and a record of safe use in foods for human or animal consumption had not yet been established nor had its use in foods been evaluated. Since then, scientific studies and evaluations regarding the use of APH(3') II in new plant development have been performed. In FDA's review of APH(3') II, FDA concluded that APH(3') II will not compromise the efficacy of antibiotic treatment, the probability of transfer of the *kan*^{r} gene consumed as a component of crops to microorganisms in the gastrointestinal tract is remote, and APH(3') II does not have any properties that would distinguish it toxicologically from any other phosphorylating enzymes in the food supply. The Nordic Council of Ministers through the Nordic Working Group on Food Toxicology and Risk Assessment (NNT) evaluated and published a report on the health aspects of marker genes in genetically engineered food plants (Ref. 2). On the basis of the information compiled for their review, Nordic Council concluded that the *kan*^{r} gene and its gene product, APH(3') II, can be considered as safe for use as a marker in the genetic tranformation of food plants.

Bejo Zaden claims that other published scientific reports and studies document the safe use of kanamycin-resistance marker genes in the development of transgenic plants produced for human consumption (Refs. 3, 4, 5 and 6). The authors of the referenced studies conclude that APH(3') II protein is readily degraded like other dietary proteins, will not compromise the efficacy of aminoglycoside antibiotics, does not possess the attributes of known protein food allergens, nor is it toxic to mammals and hence presents no risks for human or animal consumption. These authors also claim that horizontal gene transfer from transgenic plants to other organisms is very unlikely.

Combined with information from other studies, the authors of the referenced studies conclude that scientific data ~~has~~ have established that the APH(3') II protein produced in genetically engineered plants possess ~~no~~ an discernable environmental, food or feed safety concerns. Moreover, according to Bejo Zaden, the amount of Radicchio consumed by the general population is small, making it a minor component of human diets. Therefore, the presence of APH(3') II in Radicchio will not substantially increase the estimated dietary exposure of APH(3') II from its current levels of consumption. It is FDA's understanding that, in essence, Bejo Zaden has concluded that the use of APH(3') II as a processing aid in the development of Radicchio and other transgenic edible plants is safe and has provided information and published scientific literature regarding the safety of APH(3') II with their safety assessment.

Nutritional Assessment

The intent of the genetic modification made by Bejo Zaden was to produce male sterile lines of Radicchio rosso for use in the production of 100% pure hybrid seeds. Bejo Zaden did not anticipate any unintended effects from the introduction of transgenes into Radicchio. Nonetheless, to preclude any possibility that an unintended effect may have rendered the transgenic Radicchio rosso inferior for food consumption, Bejo Zaden conducted nutrient analyses on their transgenic Radicchio rosso lines, RM3-3, RM3-4 and RM3-6, and the non-transgenic control line, R3. Samples for analysis were from freeze-dried mature heads of Radicchio rosso. Three samples for each line were analyzed for sixteen parameters: moisture, crude protein, crude fat, crude fiber, total ash, acid insoluble ash, nitrogen free extract, calcium, phosphorus, magnesium, potassium, sodium, zinc, manganese, iron, and copper. The firm concludes that there are no substantial changes between transgenic Radicchio rosso and nontransgenic control plants in nutrient composition of the mature heads. The firm reports that taste tests between the genetically engineered varieties and the conventional varieties of Radicchio rosso revealed no differences in texture, taste, aftertaste and overall palatability. Tasters noted that the transgenic and the conventional varieties of Radicchio rosso were bitter. According to Bejo Zaden, this was expected since bitterness is a common characteristic of vegetable chicories.

Conclusions

Based on the safety and nutritional assessment Bejo Zaden has conducted, Bejo Zaden has concluded, in essence, that the Radicchio rosso lines containing transformation events RM3-3, RM3-4 and RM3-6 are not materially different in any respect relevant to food safety from Radicchio rosso varieties currently on the market. At this time, based on Bejo Zaden's description of its data and analysis, the agency considers Bejo Zaden's consultation on their new Radicchio rosso lines, RM3-3, RM3-4 and RM3-6, to be complete.

References

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